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Differential patterns of reactive oxygen species and antioxidative mechanisms during atrazine injury and sucrose-induced tolerance in *Arabidopsis thaliana* plantlets

Fanny Ramel[1], Cécile Sulmon[1], Matthieu Bogard[1,2], Ivan Couée[1] and Gwenola Gouesbet*[1]

Address: [1]Centre National de la Recherche Scientifique, Université de Rennes I, UMR 6553 ECOBIO, Campus de Beaulieu, bâtiment 14A, F-35042 Rennes Cedex, France and [2]INRA, UMR 1095 Génétique, Diversité et Ecophysiologie des Céréales, 234-avenue du Brezet, F-63100 Clermont-Ferrand, France

Email: Fanny Ramel - fanny.ramel@univ-rennes1.fr; Cécile Sulmon - cecile.sulmon-maisonneuve@univ-rennes1.fr; Matthieu Bogard - mbogard@clermont.inra.fr; Ivan Couée - ivan.couee@univ-rennes1.fr; Gwenola Gouesbet* - gwenola.gouesbet@univ-rennes1.fr

* Corresponding author

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Abstract

Background: Besides being essential for plant structure and metabolism, soluble carbohydrates play important roles in stress responses. Sucrose has been shown to confer to Arabidopsis seedlings a high level of tolerance to the herbicide atrazine, which causes reactive oxygen species (ROS) production and oxidative stress. The effects of atrazine and of exogenous sucrose on ROS patterns and ROS-scavenging systems were studied. Simultaneous analysis of ROS contents, expression of ROS-related genes and activities of ROS-scavenging enzymes gave an integrative view of physiological state and detoxifying potential under conditions of sensitivity or tolerance.

Results: Toxicity of atrazine could be related to inefficient activation of singlet oxygen ($^1O_2$) quenching pathways leading to $^1O_2$ accumulation. Atrazine treatment also increased hydrogen peroxide (H_2O_2) content, while reducing gene expressions and enzymatic activities related to two major H_2O_2-detoxification pathways. Conversely, sucrose-protected plantlets in the presence of atrazine exhibited efficient $^1O_2$ quenching, low $^1O_2$ accumulation and active H_2O_2-detoxifying systems.

Conclusion: In conclusion, sucrose protection was in part due to activation of specific ROS scavenging systems with consequent reduction of oxidative damages. Importance of ROS combination and potential interferences of sucrose, xenobiotic and ROS signalling pathways are discussed.

Background

Although molecular oxygen (O_2) is used as stable terminal electron acceptor in many essential metabolic processes, its partially reduced or activated forms, singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\cdot}$), are highly reactive [1]. Overproduction of these reactive oxygen species (ROS) can initiate a variety of autooxidative chain reac-

tions on membrane unsaturated fatty acids, thus yielding lipid hydroperoxides and cascades of events ultimately leading to destruction of organelles and macromolecules [2].

In plants, ROS are continuously produced as byproducts of various metabolic pathways, principally through electron transport chains in chloroplasts and mitochondria, photorespiration in peroxisomes, oxidases and peroxidases [3]. ROS, which also act as signalling molecules, have been shown to affect the expression of multiple genes [2,4], and to be involved in activation and control of various genetic stress-response programs [5].

However, numerous environmental factors such as UV-radiation, high light, drought, low or high temperature, mechanical stress and some xenobiotics disturb the prooxidant-antioxidant balance and lead to irreparable metabolic dysfunctions and cell death [6]. Different classes of herbicides are direct or indirect sources of oxidative damages in plants. The herbicide atrazine, of the triazine family, binds to the D1 protein, which results in inhibition of photosystem II (PSII) by blocking electron transfer to the plastoquinone pool [7], thus leading to production of triplet chlorophyll and $^1O_2$ [8,9].

Because of widespread use, atrazine is a common contaminant in soils, streams, rivers and lakes [10,11]. The length of water residence time associated with high loading rates results in prolonged exposure of phytoplankton communities to atrazine. Numerous studies have been carried out on the sensitivity of aquatic photosynthetic communities towards atrazine and on effects of this herbicide on reduction of photosynthesis, chlorophyll synthesis, cell growth and nitrogen fixation [12,13]. In the case of wild terrestrial plants, most studies deal with mutations of D1 protein in atrazine-resistant weeds [14], rather than with atrazine-related toxic effects.

Exogenous supply of soluble sugars, particularly sucrose, has been shown to confer to Arabidopsis plantlets a high level of atrazine tolerance [15-17]. Transcriptome profiling revealed that atrazine sensitivity and sucrose-induced atrazine tolerance were associated with important modifications of gene expression related to ROS defence mechanisms, repair mechanisms, signal transduction and cellular communication [18]. Thus, sucrose-induced atrazine tolerance was shown to depend on modifications of gene expression, which to a large extent resulted from combined effects of sucrose and atrazine. This strongly suggested important interactions of sucrose and xenobiotic signalling or of sucrose and ROS signalling, thus resulting in induction of specific transcription factors and in an integrated response to changing environmental conditions [18].

Complex arrays of detoxification mechanisms have been selected in plants against ROS accumulation and toxicity. Biochemical antioxidants, such as ascorbate, glutathione, tocopherol, flavonoids, anthocyanins and carotenoids [19,20], and ROS-scavenging enzymes, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX) and catalase (CAT) [21-23], are involved in maintaining the redox balance of cells. For example, transgenic plants with enhanced SOD activity exhibit increased tolerance to oxidative stress [22,24,25]. Moreover, Ramel et al. [18] have shown that, during sugar-induced protection against atrazine, expression of several ROS defence-related genes was enhanced.

The present work analyses the relationships between ROS patterns, expression of genes involved in synthesis of antioxidant molecules or antioxidative processes and respective enzyme activities in order to characterize atrazine sensitivity and sucrose-induced tolerance against atrazine-dependent oxidative stress. Atrazine-treated plantlets were found to exhibit an original pattern of ROS with increased levels of $^1O_2$ and H_2O_2 associated with a decrease of $O_2^{\cdot-}$ content, whereas the protective sucrose-atrazine combination favored accumulation of $O_2^{\cdot-}$ and H_2O_2. These ROS patterns were associated with differences of antioxidant gene expression and enzyme activities, thus suggesting that atrazine injuries might be due to deficient ROS-detoxification mechanisms. The possible interferences of sucrose, xenobiotic and ROS signalling are discussed.

Results

Patterns of accumulation of singlet oxygen, superoxide radical and hydrogen peroxide

The transfer of plantlets after 3 weeks of growth to control and treatment media, as described in Methods, was designed to compare plantlets at the same developmental and physiological stages. As previously described in numerous studies of sugar effects in plants, mannitol treatment was used as osmotic control. Moreover, we previously showed that the deleterious effects of atrazine on Arabidopsis plantlets followed the same dose-response curve and the same time dependence in the absence or presence of 80 mM mannitol [16,17]. It was also verified that, in accordance with previous studies [26], exogenous sugar treatment resulted in increased levels of endogenous soluble sugars in Arabidopsis plantlets (data not shown).

At the end of treatments, plantlets were specifically stained for singlet oxygen, superoxide radical, and hydrogen peroxide. Hideg et al. [27] described some limitations in the use of vacuum infiltration of ROS probes and reagents with excised leaves or leaf segments from pea, spinach or tobacco. However, vacuum infiltration has been successfully used on whole *Arabidopsis thaliana* plantlets under various experimental conditions [28-30]. Moreo-

ver, under the conditions of the present work, whatever the dye used and therefore the ROS detected, the non-stressed plantlets, transferred to 80 mM mannitol or 80 mM sucrose media, presented expected responses related to ROS production (Fig. 1, 2 and 3; Additional files 1, 2 and 3). Plantlets that were transferred for 12 h on mannitol medium presented the same ROS levels as three-week-old plantlets prior to transfer (Fig. 1, 2 and 3; Additional files 1, 2 and 3).

Detection and quantification of singlet oxygen ($^1O_2$) were performed with the specific Singlet Oxygen Sensor Green® reagent [31]. For atrazine-containing treatments (MA and SA), green fluorescence indicating primary events of $^1O_2$

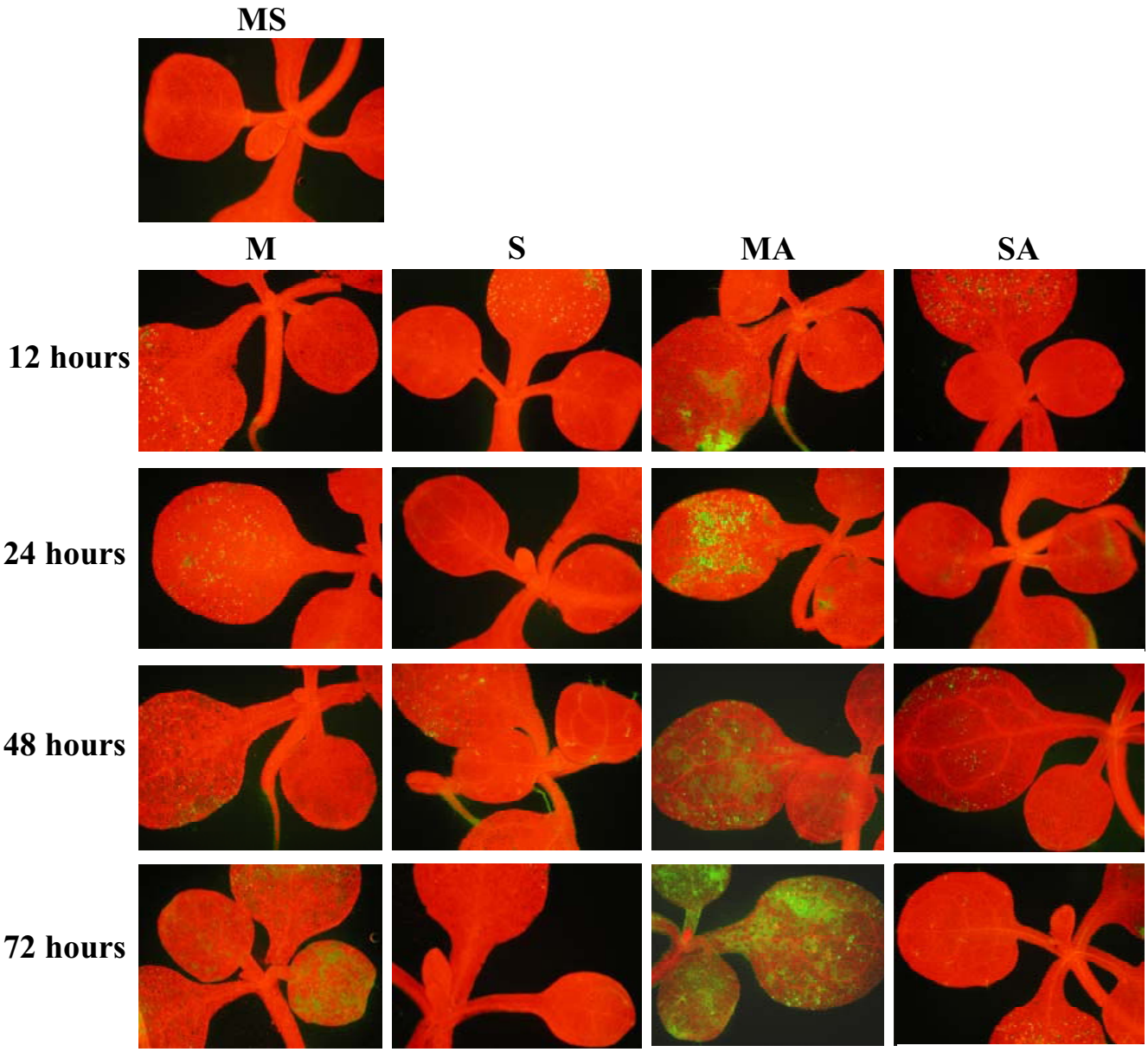

Figure 1
Visualization of singlet oxygen detected with the SOSG fluorescent probe. Detections have been done on 3-week-old MS-grown *Arabidopsis thaliana* plantlets subjected to subsequent treatment (12, 24, 48 or 72 hours) with 80 mM mannitol (M), 80 mM sucrose (S), 80 mM mannitol plus 10 μM atrazine (MA) or 80 mM sucrose plus 10 μM atrazine (SA). The fluorescence of SOSG corresponds to the green coloration, while the red color corresponds to chlorophyll autofluorescence. Green fluorescence of roots corresponds to flavonoid and porphyrin autofluorescence. Individual plantlets under the microscope are shown. Quantification of singlet oxygen is presented in Additional file 1.

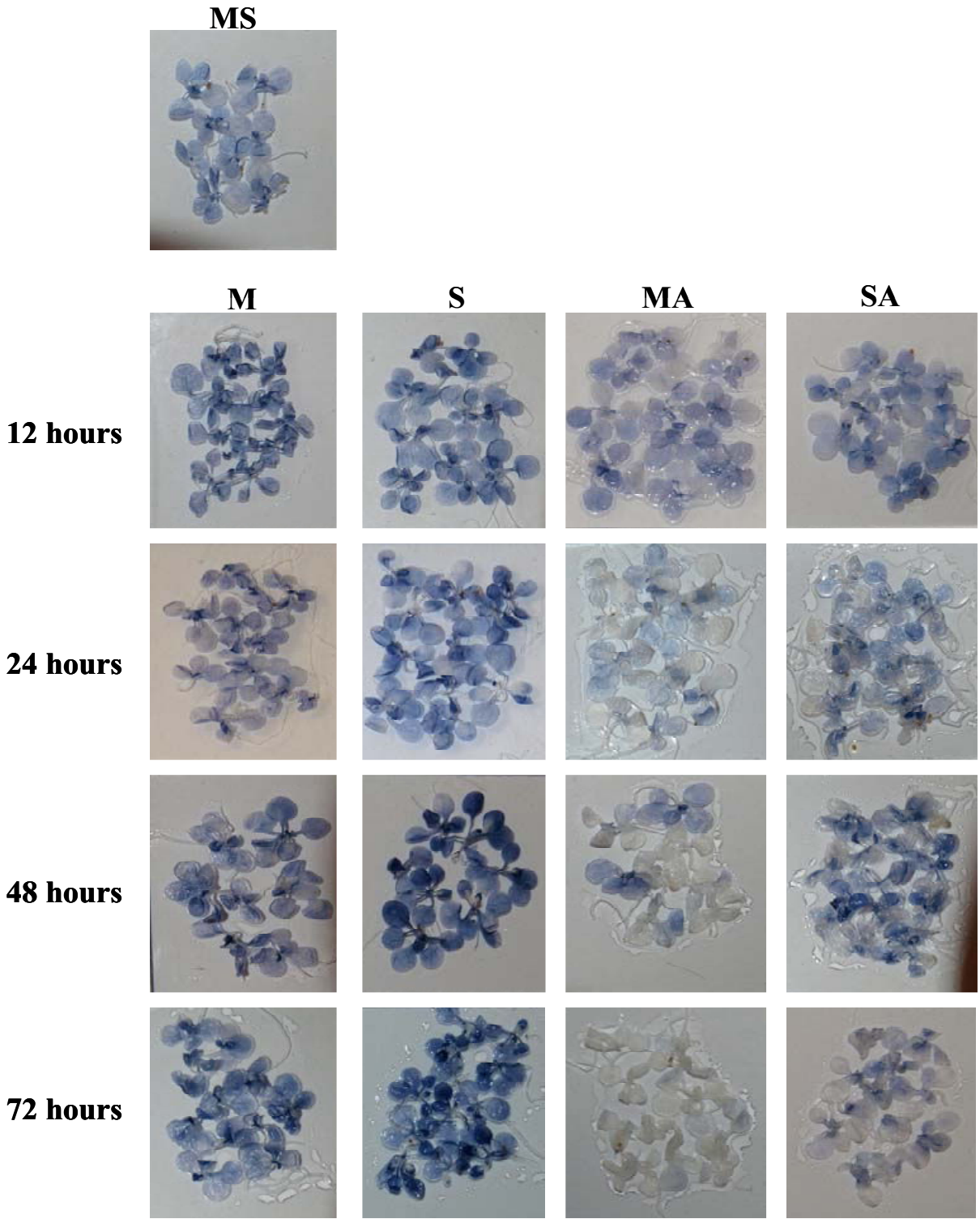

Figure 2
Visualization of superoxide radical detected by NBT staining. Detections have been done on 3-week-old MS-grown *Arabidopsis thaliana* plantlets subjected to subsequent treatment (12, 24, 48 or 72 hours) with 80 mM mannitol (M), 80 mM sucrose (S), 80 mM mannitol plus 10 μM atrazine (MA) or 80 mM sucrose plus 10 μM atrazine (SA). Groups of 15 plantlets are shown. Quantification of superoxide radical is presented in Additional file 2.

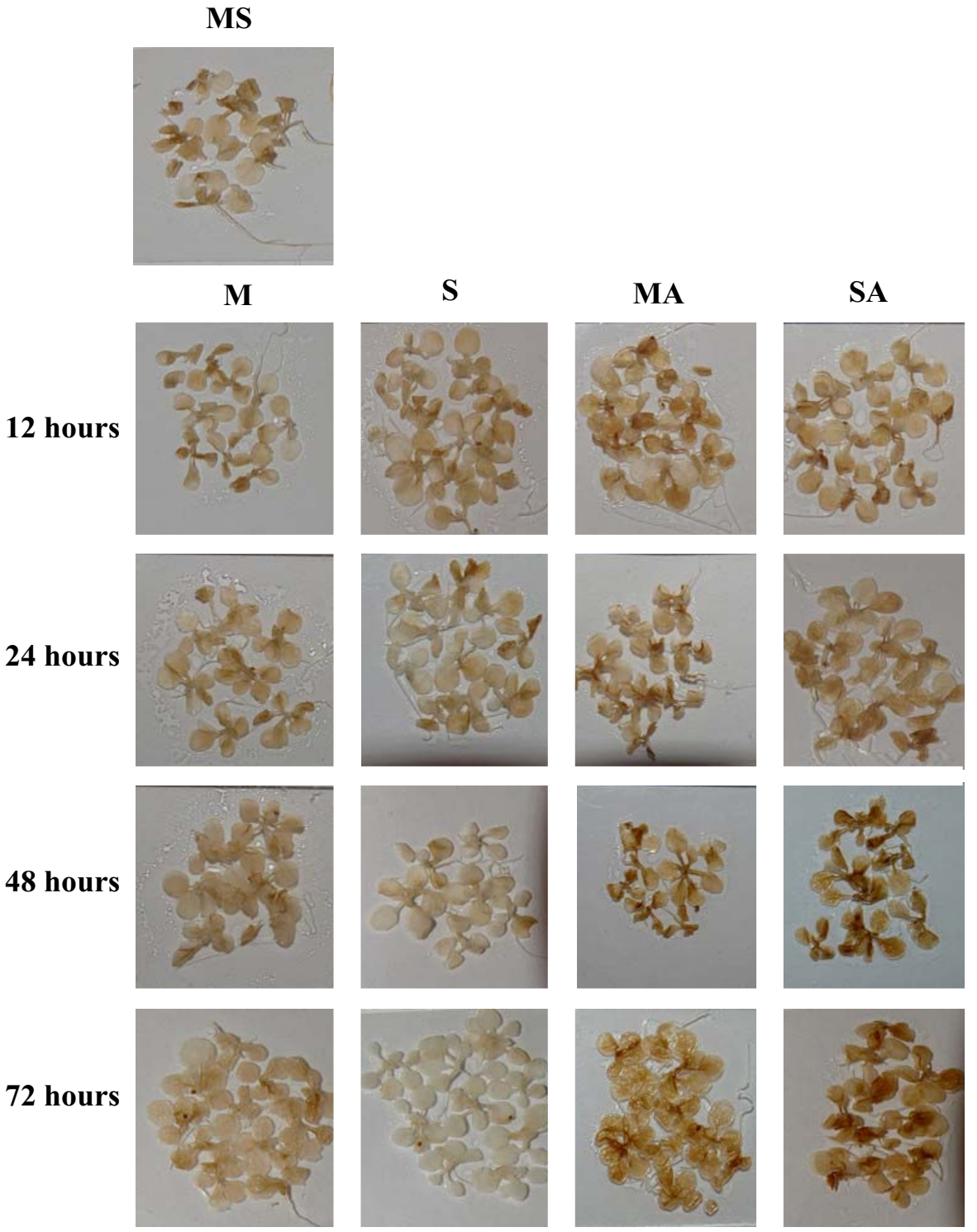

Figure 3
Visualization of hydrogen peroxide detected by DAB staining. Detections have been done on 3-week-old MS-grown *Arabidopsis thaliana* plantlets subjected to subsequent treatment (12, 24, 48 or 72 hours) with 80 mM mannitol (M), 80 mM sucrose (S), 80 mM mannitol plus 10 μM atrazine (MA) or 80 mM sucrose plus 10 μM atrazine (SA). Groups of 15 plantlets are shown. Quantification of hydrogen peroxide is presented in Additional file 3.

accumulation was detected in cotyledons as soon as after 12 hours of treatment (Fig. 1 and Additional file 1). Tolerance treatment (SA) maintained a low level of $^1O_2$ in cotyledons throughout the treatment, while atrazine treatment (MA) strongly increased $^1O_2$ production in cotyledons and leaves from 12 to 72 hours of treatment. The presence of sucrose in herbicide-containing medium thus appeared to prevent accumulation of $^1O_2$ generated by atrazine.

Superoxide radical ($O_2^{\cdot-}$) detection and quantification were performed using the nitroblue tetrazolium (NBT) staining method. The levels of superoxide radical staining after 12 hours of transfer (Fig. 2 and Additional file 2) were quite similar in the absence (M or S) or presence (MA or SA) of 10 μM atrazine. However, the time-course revealed constant levels of $O_2^{\cdot-}$ in control plantlets (M), while a strong blue coloration appeared in plantlets treated with sucrose (S). This increase was more visible in young leaves. Superoxide radical levels in atrazine-treated plantlets (MA) decreased from 24 hours of treatment. The combination of sucrose plus atrazine (SA) led to an intermediate state with slight coloration maintained in young leaves throughout the treatment. Low levels of $O_2^{\cdot-}$, relatively to the mannitol control, were also observed when a drop of 10 μM atrazine solution was directly applied to leaf tissue (data not shown).

H_2O_2 detection and quantification were performed using the 3,3'-diaminobenzidine (DAB) staining method [32]. Polymerization of DAB, visible as a brown precipitate in the presence of H_2O_2, was detected under all conditions. No coloration was observed when infiltration was carried out in the presence of ascorbic acid, thus confirming the H_2O_2 specificity of DAB staining, in accordance with previous reports [33-36]. Figure 3 and Additional file 3 summarize the time-course of H_2O_2 accumulation. From 24 hours of transfer, control (M) and sucrose-treated (S) plantlets exhibited a much weaker level of H_2O_2 than plantlets of atrazine-containing treatments (MA and SA). No variation of H_2O_2 accumulation was detected in the presence of mannitol, whereas H_2O_2 content decreased in sucrose-treated plantlets. In contrast, atrazine in the absence or presence of sucrose tended to increase progressively H_2O_2 levels until 72 hours of treatment. This increase could be detected as early as the fourth hour of atrazine treatment (data not shown). Likewise, an immediate increase of H_2O_2 levels was also observed when a drop of 10 μM atrazine solution was directly applied to leaf tissue (data not shown).

Patterns of singlet oxygen quenching mechanisms

Transcriptomic analysis showed that genes linked to the synthesis of $^1O_2$-quenchers presented contrasted patterns of expression in relation to atrazine sensitivity and tolerance (Table 1). Some genes exhibited higher transcript levels under tolerance condition (SA) and repression under atrazine injury condition (MA), thus suggesting the possibility of more efficient quenching mechanisms in the presence of sucrose. Thus, seven genes encoding thioredoxin family proteins (At2g32920, At2g35010, At2g47470, At3g06730, At4g27080, At5g42980 and At5g60640) were characterized by significant atrazine repression of expression, which was lifted by sucrose-atrazine tolerance treatment (Table 1). Only two genes encoding thioredoxin family proteins exhibited higher expression under atrazine treatment (At5g06690 and At1g08570) than under sucrose plus atrazine treatment (Table 1). In contrast, two thioredoxin genes (At1g69880 and At1g45145) and one thioredoxin reductase gene (At2g17420) were significantly induced under tolerance conditions (SA) compared to atrazine treatment (MA) (Table 1). Thioredoxins have been shown to be involved in supplying reducing power to reductases detoxifying lipid hydroperoxides or repairing oxidized proteins [37]. Thioredoxins could also act as regulators of scavenging mechanisms [38-40] and as components of signalling pathways of plant antioxidant network. Finally, Das and Das [41] presented evidence that human thioredoxin was a powerful $^1O_2$ quencher, which could protect cells and tissues against oxidative stress.

Another group of genes exhibited induction of expression under atrazine conditions, whereas they were less induced or not differentially expressed under sucrose-atrazine conditions. Activation of these genes might reflect stress signalling due to high $^1O_2$ content in atrazine treated-cells, as revealed by ROS detection ((Fig. 1 and Additional file 1). Some of these genes belonged to carotenoid biosynthesis pathways, such as Zeta-carotene desaturase *ZDS1* (At3g04870), beta-carotene hydroxylase (At4g25700) or 4-hydroxyphenylpyruvate dioxygenase *HPD* (At1g06570) (Table 1). Carotenoids, which are known to act in chloroplasts as accessory pigments in light harvesting, can detoxify $^1O_2$ and triplet chlorophyll and dissipate excess excitation energy [9].

Transcriptome profiling was carried out after 24 hours of treatment [18]. Measurements of carotenoid levels at different times of treatment showed that modifications were most contrasted after 48 hours of treatment [18]. Thus, given the potential delay between transcription and metabolic fluxes, modifications of carotenoid levels after 48 hours of treatment were compared with transcript-level modifications after 24 hours of treatment. Carotenoid (xanthophylls and carotenes) levels in *Arabidopsis thaliana* plantlets after 48 hours of treatment are presented in Table 2. Atrazine treatment tended to reduce carotenoid contents, while addition of sucrose in presence of atrazine maintained carotenoid levels near control levels. How-

Table 1: Expression of genes involved in singlet oxygen quenching after 24 hours of treatment.

Accession number	Gene description	Localisation	Log_2(ratio) Treatment comparison S/M	MA/M	SA/M
At1g08570	Thioredoxin family protein	No classification	nde	1.04	nde
At1g45145	Thioredoxin H-type 5 (TRX-H-5) (TOUL)	Cytosol	nde	nde	0.75
At1g69880	Thioredoxin, putative	No classification	2.05	nde	2.42
At2g17420	Thioredoxin reductase 2/NADPH-dependent thioredoxin reductase 2 (NTR2)	Cytoplasm	1.22	nde	1.51
At2g32920	Thioredoxin family protein	Endomembrane system	nde	-1.54	nde
At2g35010	Thioredoxin family protein	Mitochondrion	nde	-1.00	nde
At2g47470	Thioredoxin family protein	Endomembrane system	nde	-1.74	nde
At3g06730	Thioredoxin family protein	Chloroplast	nde	-0.74	nde
At4g27080	Thioredoxin family protein	Endoplasmic reticulum	nde	-0.96	nde
At5g06690	Thioredoxin family protein	Chloroplast	-1.15	1.14	nde
At5g42980	Thioredoxin H-type 3 (TRX-H-3) (GIF1)	Cytosol	nde	-0.94	nde
At5g60640	Thioredoxin family protein	Endomembrane system	nde	-1.19	nde
At1g06570	4-hydroxyphenylpyruvate dioxygenase (HPD)	Chloroplast	-0.75	3.18	2.11
At3g04870	Zeta-carotene desaturase (ZDS1)/carotene 7.8-desaturase	Chloroplast	nde	0.94	nde
At4g25700	Beta-carotene hydroxylase	Chloroplast	nde	1.07	nde
At1g08550	Violaxanthin de-epoxidase precursor. putative (AVDE1)	Photosystem II	-1.26	0.91	nde
At3g26900	Shikimate kinase family protein	Chloroplast	nde	1.69	nde
At4g36810	Geranylgeranyl pyrophosphate synthase (GGPS1)/GGPP synthetase/ farnesyltranstransferase	Chloroplast	nde	0.88	nde
At3g55610	Delta 1-pyrroline-5-carboxylate synthetase B/P5CS B (P5CS2)	Cytoplasm	0.82	3.63	2.24

Relative expressions of gene are given with their log_2(ratio) for sucrose versus mannitol (S/M), mannitol plus atrazine versus mannitol (MA/M) and sucrose plus atrazine versus mannitol (SA/M) comparison. nde: not differentially expressed. Genes with a Bonferroni *P*-value higher than 5% were considered as being not differentially expressed as described by Lurin et al. [85].

ever, carotenoid/chlorophyll ratios were not significantly different, thus indicating that the photoprotection role of carotenoids was maintained in the presence of atrazine.

Higher induction by atrazine treatment was also found for the violaxanthin de-epoxidase precursor (At1g08550) gene, which is involved in the xanthophyll cycle (Table 1). Together with carotenoids, zeaxanthin, synthesized from violaxanthin *via* the xanthophyll cycle, protects chloroplasts by accepting excitation energy from singlet chlorophyll [42]. Two genes involved in the shikimate (shikimate kinase, At3g26900) and terpenoid pathways (geranylgeranyl pyrophosphate synthase, At4g36810), which are essential for tocopherol synthesis [43], were also induced by the herbicide and not differentially expressed by the tolerance treatment (SA) (Table 1). The antioxidant tocopherol is known to scavenge oxygen free radicals, lipid peroxy radicals and $^1O_2$ [44]. Finally, the presence of atrazine alone was found to induce the At3g55610 gene, which is involved in proline synthesis, with a higher intensity than under conditions of combina-

Table 2: Carotenoid content and carotenoid/chlorophyll ratios in leaves of *Arabidopsis thaliana* plantlets after 48 hours of treatment.

Treatment	Carotenoid content (Mean ± SE) $\mu g\ g^{-1}$ FW	Carotenoid/Chlorophyll ratios
Mannitol (M)	78.6 ± 0.3	0.172 ± 0.008
Sucrose (S)	78.8 ± 0.6	0.168 ± 0.009
Mannitol atrazine (MA)	61.2 ± 0.6	0.176 ± 0.012
Sucrose atrazine (SA)	72.1± 0.8	0.186 ± 0.010

tion with sucrose (Table 1). Proline is also known to be an $^1O_2$ quencher [45].

Patterns of superoxide radical scavenging mechanisms

Excess of superoxide radical caused by numerous environmental stresses is detoxified by superoxide dismutase (SOD) enzymes and converted into H_2O_2. Seven isoenzymes have been identified, differing by their metal cofactor (Fe, Mn, or Cu and Zn), in *Arabidopsis thaliana* [46]. Transcriptome profiling was carried out after 24 hours of treatment [18]. Measurements of enzyme activities at different times of treatment showed that modifications were most contrasted after 48 hours of treatment (data not shown). Thus, given the potential delay between transcription and protein synthesis, modifications of global SOD activities after 48 hours of treatment were compared with modifications of SOD-encoding transcript levels after 24 hours of treatment.

SOD activity (Fig. 4) was decreased by atrazine treatment (MA) in comparison to the mannitol control (M). In contrast, addition of sucrose in the presence of atrazine (SA) maintained a functional level of SOD activity equivalent to that of the mannitol control. Since sucrose alone was found to increase SOD activity, it thus seemed that sucrose might balance the negative effect of atrazine in the situation of SA treatment.

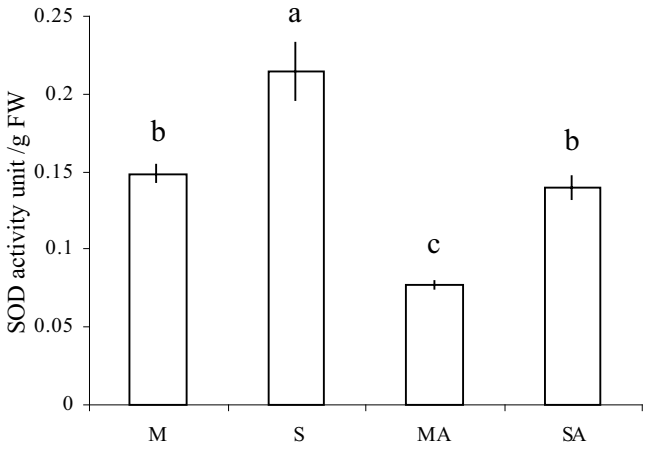

Figure 4
Effects of atrazine and sucrose on SOD enzyme activity. SOD activity was measured in protein extracts from 3-week-old MS-grown *Arabidopsis thaliana* plantlets subjected to subsequent treatment (48 hours) with 80 mM mannitol (M), 80 mM sucrose (S), 80 mM mannitol plus 10 μM atrazine (MA) or 80 mM sucrose plus 10 μM atrazine (SA). SOD activity is expressed in unit/g FW as defined in Methods. Statistical analysis was carried out as described in Methods.

Among the six isoenzyme-encoding genes represented in this microarray analysis (Table 3), three exhibited significant variations of transcript levels in comparison with control conditions, thus suggesting their potential involvement in $O_2^{\cdot-}$-detoxifying processes in relation to atrazine sensitivity and tolerance. Three genes, encoding CSD1, MSD1, FSD3, were characterized by significant repression under conditions of atrazine treatment compared to control, in accordance with the measurement of global SOD activity (Fig. 4). The *CSD1* gene (At1g08830), encoding cytosolic Cu-Zn superoxide dismutase, exhibited an induction under tolerance conditions (SA). In contrast, *MSD1* (At3g10920) and *FSD3* (At5g23310) genes, which, respectively, encode mitochondrial and chloroplastic superoxide dismutases, were not differentially expressed in the presence of sucrose. Exogenous sucrose, whether combined or not with atrazine, therefore re-established the basal level of transcripts (Table 3) and of global activity (Fig. 4), thus avoiding the repressive effects of the herbicide.

Potential origin of hydrogen peroxide accumulation in the presence of atrazine

H_2O_2 contents in atrazine-treated plantlets in the presence or absence of sucrose seemed to be independent from $O_2^{\cdot-}$ dismutation. Indeed, $O_2^{\cdot-}$ level was low in sucrose plus atrazine-treated plantlets and null in atrazine-treated plantlets. Thus, atrazine, in the absence or presence of sucrose, may promote H_2O_2-producing pathways independently from $O_2^{\cdot-}$ and $^1O_2$ accumulation. Transcriptomic analysis revealed induction of two genes encoding H_2O_2-producing enzymes in atrazine-treated plantlets in the presence or absence of sucrose (SA and MA) (Table 4): amine oxidase (At1g57770) and proline oxidase (At3g30775). Moreover, other potentially H_2O_2-producing genes were upregulated either under MA condition: a glycolate oxidase putative gene (At3g14420) and a glyoxal oxidase-related gene (At3g53950); or under SA condition: two genes encoding acyl-CoA oxidases (At4g16760, At5g65110) (Table 4).

Patterns of hydrogen peroxide scavenging mechanisms

In order to investigate the efficiency of hydrogen peroxide scavenging mechanisms, global H_2O_2-scavenging enzyme activities and transcript levels of related genes were analysed. As explained above, modifications of enzyme activities after 48 hours of treatment were compared with modifications of transcript levels after 24 hours of treatment.

H_2O_2 can be principally scavenged by two different ways: ascorbate-glutathione cycles and catalases, which play important roles in plant defence and senescence. Ascorbate-glutathione cycles are catalysed by a set of four enzymes: ascorbate peroxidase (APX), monodehy-

Table 3: Expression of genes encoding enzymes involved in $O_2^{\cdot-}$ scavenging after 24 hours of treatment.

Accession number	Gene description	Localisation	Log_2(ratio) Treatment comparison S/M	MA/M	SA/M
At1g08830	Superoxide dismutase (Cu-Zn) (SODCC)/copper/zinc superoxide dismutase (CSD1)	Cytoplasm	0.80	-0.70	1.22
At2g28190	Superoxide dismutase (Cu-Zn). chloroplast (SODCP)/copper/zinc superoxide dismutase (CSD2)	Chloroplast	-0.73	nde	-0.76
At3g10920	Superoxide dismutase (Mn). mitochondrial (SODA)/manganese superoxide dismutase (MSD1)	Mitochondrion	nde	-1.23	nde
At4g25100	Superoxide dismutase (Fe). chloroplast (SODB)/iron superoxide dismutase (FSD1)	Chloroplast	nde	nde	nde
At5g18100	Superoxide dismutase (Cu-Zn)/copper/zinc superoxide dismutase (CSD3)	Peroxisome	nde	nde	nde
At5g23310	Superoxide dismutase (Fe)/iron superoxide dismutase 3 (FSD3)	Chloroplast	nde	-1.34	nde

Relative expressions of gene are given with their log_2(ratio) for sucrose versus mannitol (S/M), mannitol plus atrazine versus mannitol (MA/M) and sucrose plus atrazine versus mannitol (SA/M) comparison. nde: not differentially expressed. Genes with a Bonferroni *P*-value higher than 5% were considered as being not differentially expressed as described by Lurin et al. [85].

droascorbate reductase (MDAR), glutathione-dependent dehydroascorbate reductase (DHAR), and glutathione reductase (GR) [47].

The five enzymes belonging to H_2O_2-scavenging mechanisms presented two different profiles of global activity according to the different treatments. The majority of enzymes involved in ascorbate-glutathione cycles (APX, DHAR and MDAR) were differentially affected by the different treatments. Activity of these three enzymes was significantly reduced by addition of atrazine, while sucrose treatment had an opposite effect and significantly increased these activities (Fig. 5a, b, c). The tolerance condition (SA) succeeded to limit repressive effects of the herbicide and maintained enzyme activities at the control level. The fourth enzyme of the ascorbate-glutathione cycles, GR, did not present any significant variation of activity between the different treatments (Fig. 5d). Finally, catalase exhibited slightly lower activity under conditions of sucrose plus atrazine, when compared to control and atrazine-containing medium (Fig. 5e).

The repressive effect of atrazine in the absence of sucrose (MA treatment) on APX global activity was correlated with a general repression of *APX* genes (Fig. 5a, Table 5). Among the six *APX* genes present in the microarray, the cytosolic *APX1* (At1g07890), the stromal *sAPX* (At4g08390) and the chloroplastic *APX4* (At4g09010) genes exhibited important decrease of transcript levels under conditions of atrazine treatment (MA) compared to mannitol control, while the other *APX* genes were not differentially expressed in the presence of atrazine. Whereas *APX4* expression remained downregulated in the presence of sucrose plus atrazine, this tolerant condition balanced the repressive effects of atrazine for *APX1* and *sAPX* genes, which recovered a level of transcript similar to the control. Finally, and in contrast with global APX activity, the thylakoid-bound *tAPX* (At1g77490) gene was not affected by

Table 4: Expression of genes potentially encoding H_2O_2-producing enzymes after 24 hours of treatment.

Accession number	Gene description	Localisation	Log_2(ratio) Treatment comparison S/M	MA/M	SA/M
At1g57770	Amine oxidase family	Chloroplast	nde	1.59	0.80
At3g14420	(S)-2-hydroxy-acid oxidase, peroxisomal, putative/glycolate oxidase, putative/short chain alpha-hydroxy acid oxidase, putative	Peroxisome	-1.08	1.33	nde
At3g30775	Proline oxidase, mitochondrial/osmotic stress-responsive proline dehydrogenase (POX) (PRO1) (ERD5)	Mitochondrion	nde	2.51	1.22
At3g53950	Glyoxal oxidase-related	Endomembrane system	nde	1.00	nde
At4g16760	Acyl-CoA oxidase (ACX1)	Peroxisome	0,87	nde	1.48
At5g65110	Acyl-CoA oxidase (ACX2)	Peroxisome	0.91	nde	1.83

Relative expressions of gene are given with their log_2(ratio) for sucrose versus mannitol (S/M), mannitol plus atrazine versus mannitol (MA/M) and sucrose plus atrazine versus mannitol (SA/M) comparison. nde: not differentially expressed. Genes with a Bonferroni *P*-value higher than 5% were considered as being not differentially expressed as described by Lurin et al. [85].

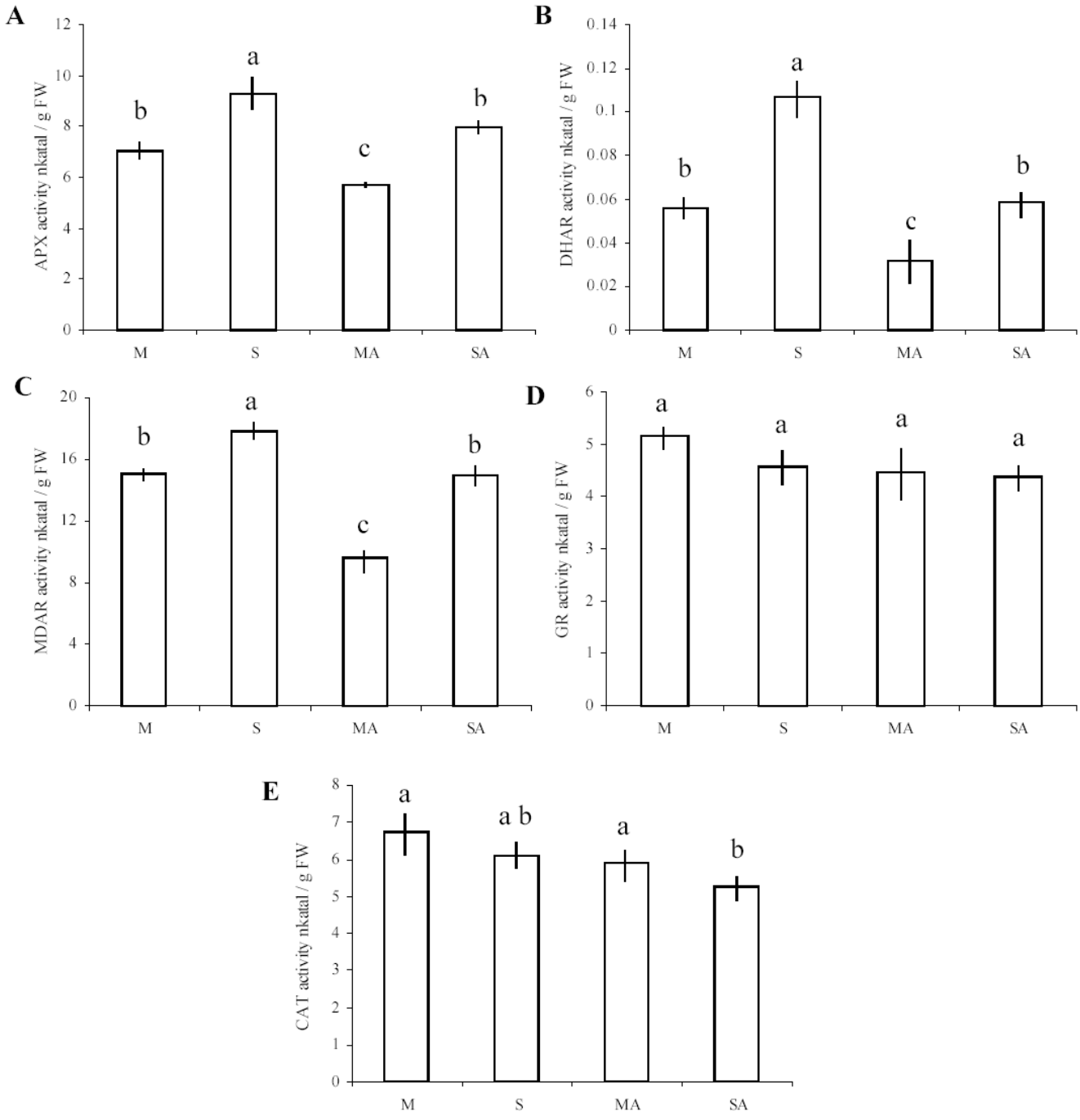

Figure 5

Effects of atrazine and sucrose on antioxidative enzyme activities. Activities of ascorbate peroxidase (APX) (A), dehydroascorbate reductase (DHAR) (B), monodehydroascorbate reductase (MDAR) (C), glutathione reductase (GR) (D) and catalase (CAT) (E) were measured in protein extracts from 3-week-old MS-grown *Arabidopsis thaliana* plantlets subjected to subsequent treatment (48 hours) with 80 mM mannitol (M), 80 mM sucrose (S), 80 mM mannitol plus 10 μM atrazine (MA) or 80 mM sucrose plus 10 μM atrazine (SA). Enzymatic activities are expressed in nkatal/g FW, nkatal corresponds to the amount of enzymatic activity that catalyzes the transformation of one nmole of substrate per second. Statistical analysis was carried out as described in Methods.

Table 5: Expression of genes encoding enzymes involved in ascorbate-glutathione cycles after 24 hours of treatment.

Accession number	Gene description	Localisation	Log_2(ratio)		
			Treatment Comparison		
			S/M	MA/M	SA/M
At1g07890	L-ascorbate peroxidase 1. cytosolic (APX1)	Cytosol	nde	-1.92	nde
At1g77490	L-ascorbate peroxidase. thylakoid-bound (tAPX)	Chloroplast	-1.03	nde	-1.08
At3g09640	L-ascorbate peroxidase 2 (APX2)	Cytoplasm	nde	nde	nde
At4g08390	L-ascorbate peroxidase. stromal (sAPX)	Chloroplast	1.46	-1.18	nde
At4g09010	L-ascorbate peroxidase 4 (APX4)	Chloroplast	-0.79	-1.12	-1.40
At4g35000	L-ascorbate peroxidase 3 (APX3)	Peroxisome	nde	nde	nde
At1g75270	Dehydroascorbate reductase (DHAR2)	Cytoplasm	1.92	-0.91	2.70
At5g16710	Dehydroascorbate reductase (DHAR3)	Chloroplast	nde	nde	nde
At5g36270	Dehydroascorbate reductase. putative	Cytoplasm	0.80	nde	1.20
At1g63940	Monodehydroascorbate reductase (MDAR5)	Chloroplast	nde	nde	nde
At3g09940	Monodehydroascorbate reductase (MDAR3)	Cytoplasm	nde	nde	nde
At3g27820	Monodehydroascorbate reductase (MDAR4)	Cytoplasm	nde	nde	nde
At3g52880	Monodehydroascorbate reductase (MDAR1)	Cytoplasm	nde	nde	nde
At5g03630	Monodehydroascorbate reductase (MDAR2)	Cytoplasm	nde	-1.35	1.11
At3g24170	Glutathione reductase. putative (GR1)	Cytoplasm	1.15	nde	0.92
At3g54660	Gluthatione reductase. chloroplast (GR2)	Chloroplast	nde	nde	nde

Relative expressions of gene are given with their log_2(ratio) for sucrose versus mannitol (S/M), mannitol plus atrazine versus mannitol (MA/M) and sucrose plus atrazine versus mannitol (SA/M) comparison. nde: not differentially expressed. Genes with a Bonferroni *P*-value higher than 5% were considered as being not differentially expressed as described by Lurin et al. [85].

atrazine, while sucrose repressed its expression under S and SA conditions.

Dehydroascorbate reductase (DHAR) is a key component of the ascorbate recycling system. DHAR recycles dehydroascorbate into ascorbate by using reduced glutathione as a reductant. Two functional *DHAR* genes, among three that are encoded in the *Arabidopsis thaliana* genome, plus a putative gene, were represented in the microarray (Table 5). The cytosolic *DHAR2* (At1g75270) and putative *DHAR* (At5g36270) genes exhibited high induction in the combined presence of sucrose and atrazine, and, respectively, a slight repression or no variation in the presence of atrazine in comparison to control condition. In contrast to the repressive effects of atrazine, which were associated with a decrease of DHAR activity, the increase of *DHAR* transcript levels in the combined presence of sucrose and atrazine was not associated with an increase of global DHAR enzyme activity (Fig. 5b).

Reduction of monodehydroascorbate by monodehydroascorbate reductase (MDAR) is also an important step in ascorbate recycling. Among the five *MDAR* genes present in the microarray, only cytosolic *MDAR2* (At5g03630) exhibited differential expression patterns according to the treatment applied. While atrazine repressed its expression, the protective combination of sucrose and atrazine upregulated it (Table 5). Atrazine was also found to decrease global MDAR activity, while sucrose plus atrazine treatment resulted in maintenance of MDAR activity relatively to the mannitol control (Fig. 5c).

Glutathione serves as a reductant in oxidation-reduction processes, such as recycling of oxidised ascorbate by dehydroascorbate reductase [48]. Reduction of oxidised glutathione is catalysed by glutathione reductase (GR), which requires NADPH. Among the two isoenzymes present in the microarray, only the cytosolic glutathione reductase *GR1* (At3g24170) was found to be induced by sucrose-atrazine and sucrose treatments, while no variation of expression was detected in the presence of atrazine (Table 5). These variations of expression were not associated with changes of global GR activity, since no significant difference of activity was observed between treatments (Fig. 5d).

The second way to reduce H_2O_2 content in cells is activation of catalases (CAT), which catalyse dismutation of H_2O_2 into water and oxygen [49]. Little variation of transcript levels was detected for the three catalase isoenzymes (Table 6). *CAT2* (At4g35099) exhibited upregulation by atrazine stress, while *CAT3* was slightly downregulated by the protective sucrose plus atrazine treatment. In relation

Table 6: Expression of genes encoding enzymes involved in H_2O_2 scavenging after 24 hours of treatment.

Accession number	Gene description	Localisation	Log_2(ratio)		
			Treatment comparison		
			S/M	MA/M	SA/M
At1g20620	Catalase 3	Peroxisome	nde	nde	-0.74
At1g20630	Catalase 1	Peroxisome	nde	nde	nde
At4g35090	Catalase 2	Peroxisome	nde	0.96	nde

Relative expressions of gene are given with their log_2(ratio) for sucrose versus mannitol (S/M), mannitol plus atrazine versus mannitol (MA/M) and sucrose plus atrazine versus mannitol (SA/M) comparison. nde: not differentially expressed. Genes with a Bonferroni *P*-value higher than 5% were considered as being not differentially expressed as described by Lurin et al. [85].

with these slight changes of transcript levels (Table 6), global catalase activities were found to show little variation (Fig. 5e).

Discussion

Characterisation of the impact of atrazine on ROS patterns

ROS patterns appear to depend strongly on the nature and intensity of stress conditions applied to plants [50]. It is therefore of great importance to characterise ROS accumulation kinetics associated with a particular stress, and not to rely on expected effects. Thus, while, as expected, atrazine inhibition of photosystem II was associated with $^1O_2$ accumulation [7] (Fig. 1 and Additional file 1), decrease of superoxide radical levels and increase of H_2O_2 levels were also observed (Figs. 2, 3 and Additional files 2, 3). This disagreed with the proposed, but experimentally unproven, accumulation of superoxide radical by triazine treatment in Arabidopsis leaves [51]. It was however coherent with inhibition of photosynthetic activity and of the Mehler reaction, whereby superoxide radical is formed by reduction of oxygen at the PSI site [52]. Atrazine binding to D1 protein of PSII and inhibition of electron feeding to PSI were indeed likely to decrease superoxide radical production by blocking the Mehler reaction.

The induction of H_2O_2 accumulation by atrazine was all the more surprising as it occurred rapidly after transfer to atrazine (Fig. 3 and Additional file 3) and in the absence or in the presence of sucrose, which by itself had a negative effect on H_2O_2 accumulation. This is, to our knowledge, the first demonstration of rapid *in vivo* H_2O_2 accumulation under conditions of atrazine treatment. The negative effect of sucrose on H_2O_2 accumulation was consistent with the previously-described repression of protein and lipid catabolism, including a number of oxidase-based processes, by soluble sugars [18,53]. In contrast, atrazine by itself was found to induce a number of genes encoding oxidases, the most highly induced being a gene encoding a proline oxidase (Table 4). Since this induction occurred prior to significant impairment of photosystems and phototrophic growth [18], it could not be ascribed to a situation of metabolic starvation. Activation of protein and lipid catabolism and of oxidase-based processes has been reported to occur under conditions of carbohydrate limitation or starvation [54,55]. In this context, it was extremely interesting that, in the presence of exogenous sucrose, i.e. in a situation of carbohydrate optimum, atrazine was able to induce a number of oxidase-encoding genes and other genes typical of carbohydrate-limitation response, such as the gene encoding isovaleryl-CoA dehydrogenase [18,56].

Numerous abiotic stressors, including xenobiotics, are known to produce oxidative stress in photosynthetic organisms. This is the case for benzoxazolinone [57], metronidazole, dinoterb [58], acetochlor [59], copper [60], wounding [61] and high light [6]. Studies on these stresses mainly focus on the effects of a single ROS and rarely consider the effects of ROS combination. However, ROS are chemically distinct and selectively perceived for the fine control of adjusting antioxidants and photosynthesis to different environmental stress conditions [62]. Indeed, cross-talk between $^1O_2$ and H_2O_2 has been clearly demonstrated by Laloi et al. [50], who suggested antagonistic interactions between $^1O_2$ and H_2O_2 with a reduction of $^1O_2$-mediated cell death and stress signalling response by H_2O_2 content. In contrast, the present condition of atrazine treatment, which eventually leads to plantlet death, was characterised by high $^1O_2$, high H_2O_2 and low superoxide radical levels. Laloi et al. [63], who described antagonistic effects between $^1O_2$, and H_2O_2, modulated H_2O_2 levels in Arabidopsis transgenic plants at the plastid level. It was thus possible that non-antagonistic effects of H_2O_2 and $^1O_2$ under conditions of atrazine treatment were due to differences of ROS localisation.

Finally, among the set of 29 induced transcription factors that have been characterized as $^1O_2$-specific by Gadjev et al. [64], only one was slightly induced (data not shown) during the course of atrazine treatment despite the high accumulation of singlet oxygen (Fig. 1 and Additional file 1). The analysis of $^1O_2$ responses by Gadjev et al. [64] was based on studies of the Arabidopsis conditional *flu*

mutant [65]. It was thus clear that other signals than $^1O_2$ were perceived by atrazine-treated plantlets or that atrazine-induced $^1O_2$ accumulation involved other processes and responses than *flu*-mutant-dependent $^1O_2$ accumulation [50,64,65]. However, full characterisation of the signalling events associated with xenobiotic exposure in plants remains to be carried out.

Impairment of antioxidant defences in the presence of atrazine

Atrazine-treated plantlets were characterised by low $O_2^{\cdot-}$ levels and high H_2O_2 levels, in contrast with sucrose-treated atrazine-tolerant plants, which showed high $O_2^{\cdot-}$ and high H_2O_2 levels. These differences of ROS patterns were associated with striking differences of gene expressions and enzyme activities involved in ROS-scavenging pathways.

Thus, atrazine sensitivity was associated with down-regulation of key players of H_2O_2 scavenging. Among the four enzymes involved in ascorbate-glutathione cycles, which are essential to remove large amounts of H_2O_2 generated by stress [48,66], three enzymes (APX, MDAR and DHAR) exhibited a significant decrease of global activities in atrazine-treated plantlets. Moreover, this repression was correlated with a global down-regulation of typical corresponding transcripts (*APX1*, *sAPX*, *DHAR2*, and *MDAR2*), which, conversely, have already been shown to undergo important induction during responses to several environmental abiotic stresses. APX1, a cytosolic enzyme, has previously been described as a central component of the reactive oxygen gene network of Arabidopsis [67]. Involvement of sAPX in response to oxidative stress has also been reported by transcriptional induction in the presence of H_2O_2, methylviologen, $FeCl_3$ or UV treatments in soybean seedlings [68]. Finally, Yoshida et al. [69] reported the importance of DHAR2 under conditions of ozone treatment, with higher sensitivity to ozone in a *DHAR2*-deficient mutant, probably due to insufficient recycling of ascorbate.

Consequently, repression of these transcripts and decrease of the corresponding enzyme activities in the presence of atrazine might accentuate the effects of H_2O_2 accumulation by reduction of ascorbate recycling, thus leading to disruption of antioxidant mechanisms and propagation of atrazine injuries. It was thus clear that the effects of atrazine at transcript level [18] had actual negative consequences on biochemical defences and could be involved in xenobiotic sensitivity. This is strong evidence that xenobiotic sensitivity may be linked to gene regulation effects in plants. Correlatively, the situation of sucrose-induced tolerance was characterised by the lifting of atrazine repression, in the case of *APX1* and *sAPX*, or by the induction by sucrose-atrazine combination, in the case of *DHAR2* and *MDAR2*. These positive effects on transcript levels were associated with maintenance of the corresponding enzyme activities at control levels. Although ROS can mediate induction of protective proteins involved in the stability of specific mRNAs [70], they can also cause RNA oxidative damages and induce protein inactivation and degradation [71]. Increase of transcript levels was therefore likely to be an adaptive response to ensure protein synthesis under stress conditions resulting in higher protein turnover.

The decline of $O_2^{\cdot-}$ levels in atrazine-treated plantlets, which, as explained above, could be ascribed to inhibition of electron transfer through PSI, was associated with a general repression of transcripts encoding the different isoenzymes of SOD and with a decrease of the global activity of this $O_2^{\cdot-}$-scavenging enzyme family, thus indicating that atrazine-treated cells responded to the low superoxide radical situation. Association of low $O_2^{\cdot-}$ and high H_2O_2 may be a cause for the ill-adapted response of anti-oxidant defences in atrazine-treated plantlets, thus suggesting that further work should be carried out on the adaptation of organisms to fluctuations of ROS combinations.

Mechanisms of sucrose-induced tolerance to singlet oxygen

In contrast with non-induction of H_2O_2-scavenging systems, atrazine-treated plantlets seemed to be able to sense the increase of $^1O_2$ levels and induce some genes potentially involved in $^1O_2$ quenching (Table 1). Thus, atrazine-treated plantlets, in the absence or presence of sucrose showed increased expression of 4-Hydroxyphenylpyruvate dioxygenase (HPD) gene (At1g06570), which could be involved in the maintenance of the photoprotective role of carotenoids. The At5g06690 gene, encoding a chloroplastic thioredoxin, which is a potential $^1O_2$-quencher [41], was also induced in atrazine-treated plantlet. However, generally, the thioredoxin gene family was negatively affected by atrazine treatment, with 7 genes among 12 significantly repressed by atrazine. Correlatively, ten of these twelve genes showed lifting of repression or significant induction in the combined presence of sucrose and atrazine. Nevertheless, most of these genes encoded extraplastidial thioredoxins or thioredoxins of unknown localisation (Table 1). The link of thioredoxin gene family differential expression with efficient $^1O_2$ quenching in the presence of atrazine plus sucrose (Fig. 1 and Additional file 1) was thus difficult to ascertain. On one hand, several studies have shown the efficiency of thioredoxins in maintenance of cellular reductant environment and in cytoprotective mechanisms [37-40]. On the other hand, efficient $^1O_2$ quenching in the case of PSII inhibition by atrazine would require the involvement of chloroplastic TRXs. Two TRX genes, At3g06730 and At5g06690, have been

described as encoding chloroplastic TRXs (Table 1). Expression of these two genes showed contrasted patterns in the presence of atrazine or in the presence of sucrose plus atrazine, with At3g06730 being repressed by atrazine, and At5g06690 being induced by atrazine, whereas the presence of sucrose and atrazine resulted in a return to baseline levels. Thus, further work would be required to analyse the physiological significance of this different pattern, and whether the At3g06730 gene product may play an important role in atrazine responses. Further work would also be required to characterise the potential importance of At1g69880 and At2g17420 TRX genes, which are induced by sucrose and by sucrose plus atrazine, in sucrose-induced tolerance.

Conclusion

Parallel and integrative analysis therefore revealed correlated modifications of ROS patterns, antioxidant biochemical defences, and corresponding transcript markers, under conditions of atrazine sensitivity and of sucrose-induced tolerance. Atrazine injury was shown to be related with increased levels of singlet oxygen and hydrogen peroxide in leaves. Sucrose-treated plantlets were able to sense changing ROS levels and activate efficient quenching and antioxidant systems, whereas, in the absence of sucrose protection, atrazine-treated plantlets failed to develop fully these defence mechanisms. It thus seemed that atrazine may generate signals that activate some H_2O_2-producing pathways, and that impair the induction and activation of antioxidant defence mechanisms. Further work is needed to characterise completely the complex signalling events associated with xenobiotic exposure in plants.

Methods

Plant material and growth conditions

Seeds of *Arabidopsis thaliana* (ecotype Colombia, Col0) were surfaced-sterilized in bayrochlore/ethanol (1/1, v/v), rinsed in absolute ethanol and dried overnight. Germination and growth were carried out under axenic conditions in square Petri dishes. After seeds were sowed, Petri dishes were placed at 4°C for 48 h in order to break dormancy and homogenize germination and transferred to a control growth chamber at 22°C under a 16 h light period regime at 85 μmol m^{-2} s^{-1} for 3 weeks. Growth medium consisted of 0.8% (w/v) agar in 1× Murashige and Skoog (MS) basal salt mix (M5519, Sigma-Aldrich) adjusted to pH 5.7. Plantlets were then transferred to fresh MS agar medium containing 80 mM mannitol (M, control), 80 mM mannitol and 10 μM atrazine (MA, lethal treatment), 80 mM sucrose (S, sugar treatment) and 80 mM sucrose and 10 μM atrazine (SA, tolerance treatment).

Chlorophyll and carotenoid extraction and quantification

Pigments were extracted by pounding aerial parts of seedlings in 80% acetone, and absorbance of the resulting extracts was measured at 663 nm, 646 nm and 470 nm. Levels of chlorophyll and total carotenoids (xanthophylls and carotenes) were determined from the equations given by Lichtenthaler and Wellburn [72]. Measurements were done on 3 replicas of 5–10 pooled seedlings each.

Singlet oxygen staining

Three week-old plantlets were transferred for 12, 24, 48 or 72 hours to the different control and treatment media described above (M, S, MA and SA). Plantlets, prior to the transfer and at the end of the treatment, were immersed and infiltrated in the dark under vacuum with a solution of 100 μM Singlet Oxygen Sensor Green® reagent (SOSG) (S36002, Invitrogen) [31] in 50 mM phosphate potassium buffer (pH 7.5). Infiltrated plantlets were then placed again on control and treatment media during 30 minutes in the light before being photographed under the microscope. Following excitation at 480 nm, the fluorescence emission at 530 nm was then detected by an Olympus BX41 spectrofluorometer coupled with a camera. The presence of red chlorophyll autofluorescence from chloroplasts did not alter the green fluorescence of SOSG. The infiltration method was chosen in order to measure singlet oxygen levels after the different times of treatment. Image analysis and quantification of level fluorescence were performed using the ImageJ software [73]. Experiments were repeated four times on at least 15 plantlets.

Superoxide radical staining

The nitroblue tetrazolium (NBT) (N6876, Sigma-Aldrich) staining method of Rao and Davis [74] was modified as follows for *in situ* detection of superoxide radical. Three week-old plantlets were transferred for 12, 24, 48 or 72 hours to the different control and treatment media described above (M, S, MA and SA). Plantlets, prior to the transfer and at the end of the treatment, were immersed and infiltrated under vacuum with 3.5 mg ml^{-1} NBT staining solution in potassium phosphate buffer (10 mM) containing 10 mM NaN_3. After infiltration, stained plantlets were bleached in acetic acid-glycerol-ethanol (1/1/3) (v/v/v) solution at 100°C during 5 min. Plantlets were then stored in a glycerol-ethanol (1/4) (v/v) solution until photographs were taken. $O_2^{\cdot-}$ was visualized as a blue color produced by NBT precipitation. A modified version of previously described assays for superoxide quantification was used [75,76]. Briefly, NBT-stained plantlets were ground in liquid nitrogen, the formazan content of the obtained powder was solubilized in 2 M KOH-DMSO (1/1.16) (v/v), and then centrifuged for 10 min at 12,000 *g*. The A_{630} was immediately measured, and compared with a standard curve obtained from known amounts of NBT

in the KOH-DMSO mix. Experiments were repeated four times on at least 15 plantlets.

Hydrogen peroxide staining

The H_2O_2 staining agent, 3,3'diaminobenzidine (DAB) (D5637, Sigma-Aldrich), was dissolved in H_2O and adjusted to pH 3.8 with KOH. The DAB solution was freshly prepared in order to avoid auto-oxidation [32]. Three week-old plantlets were transferred for 12, 24, 48 or 72 hours to the different control and treatment media described above (M, S, MA and SA). Plantlets, prior to the transfer and at the end of the treatment, were immersed and infiltrated under vacuum with 1.25 mg ml^{-1} DAB staining solution. Stained plantlets were then bleached in acetic acid-glycerol-ethanol (1/1/3) (v/v/v) solution at 100°C during 5 min, and then stored in glycerol-ethanol (1/4) (v/v) solution until photographs were taken. H_2O_2 was visualized as a brown color due to DAB polymerization. Quantification of H_2O_2 contents was determined using the method of Kotchoni et al. (2006) [77]. The DAB-stained plantlets were ground in liquid nitrogen. The resulting powder was homogenized in 0.2 M $HClO_4$, and then centrifuged for 10 min at 12,000 *g*. The A_{450} was immediately measured and compared with a standard curve containing known amounts of H_2O_2 in 0.2 M $HClO_4$-DAB. Experiments were repeated four times on at least 15 plantlets. The specificity of DAB staining towards H_2O_2 was assessed in control infiltrations in the presence of 10 mM ascorbic acid.

Enzyme activities

Three week-old plantlets were transferred for 48 hours to the different control and treatment media described above (M, S, MA and SA). Whole plantlets (100 mg FW) were ground in liquid nitrogen to extract total proteins. The powder obtained was suspended in 500 µl of extraction buffer containing 50 mM phosphate buffer (pH 7.5), 1% (w/v) polyvinylpyrrolidone (PVP), 0.5% (v/v) Triton X-100, 1 mM EDTA and a cocktail of protease inhibitors (P9599, Sigma-Aldrich). In the specific case of APX activity measurement, the plant powder was suspended in 50 mM Hepes (pH 7) buffer containing 0.5 mM ascorbate, 0.5% (v/v) Triton X-100 and 1% (w/v) PVP. After centrifugation (15 min, 10,000 *g*), the supernatant was recovered and a second extraction of the pellet was identically realized. The two supernatants were pooled and constituted the total protein extract that was immediately used for enzyme activity measurement.

Superoxide dismutase (SOD) activity (EC 1.15.1.1) was determined using the method of Beauchamp and Fridovich [78] that spectrophotometrically measures inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm. One unit of SOD activity was defined as the amount of enzyme required to inhibit the reduction rate of NBT by 50%. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.5), 10 mM methionine, 2 µM riboflavin, 0.1 mM EDTA, 70 µM NBT and enzyme sample. Reactions were carried out at 25°C under a light intensity of about 120 µmol m^{-2} s^{-1} for 10 min.

Ascorbate peroxidase (APX) activity (EC 1.11.1.11) was measured according to Nakano and Asada [79] by monitoring the rate of hydrogen peroxide-dependent oxidation of ascorbate at 290 nm (E = 2.8 mM^{-1} cm^{-1}). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7), 0.5 mM ascorbic acid, 0.1 mM H_2O_2, 1 mM EDTA and enzyme sample.

Dehydroascorbate reductase (DHAR) activity (EC 1.8.5.1) was measured as described by Hossain and Asada [80]. DHAR was assayed spectrophotometrically by monitoring the increase in absorbance at 265 nm due to ascorbate formation (E = 14 mM^{-1} cm^{-1}). The reaction mixture, freshly prepared in N_2-saturated buffer, consisted of 50 mM potassium phosphate buffer (pH 7), 0.5 mM dehydroascorbate, 5 mM reduced glutathione, 1 mM EDTA and enzyme sample. Correction was made for non-enzymatic reduction rate of DHA in absence of protein extract.

Monodehydroascorbate reductase (MDAR) activity (EC 1.6.5.4) was measured as described by Hossain et al. [81]. MDAR was assayed spectrophotometrically by following the decrease in absorbance at 340 nm due to NADH oxidation (E = 6.2 mM^{-1} cm^{-1}). The reaction mixture consisted of 50 mM buffer TES (pH 7.5), 0.1 mM NADH, 2.5 mM ascorbate, ascorbate oxidase (1 U ml^{-1}) (Curcubita enzyme (EC 1.10.3.3), A0157, Sigma-Aldrich) and enzyme sample.

Glutathione reductase (GR) activity (EC 1.6.4.2) was measured as described by Smith et al. [82] following spectrophotometrically the disappearance of NADPH at 340 nm (E = 6.2 mM^{-1} cm^{-1}). The reaction mixture contained 50 mM Hepes-NaOH buffer (pH 7.5), 0.5 mM oxidized glutathione, 0.25 mM NADPH, 0.5 mM EDTA and enzyme sample.

Catalase (CAT) activity (EC 1.11.1.6) was measured spectrophotometrically at 250 nm by following the disappearance of H_2O_2 (E = 39.4 mM^{-1} cm^{-1}) in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7) and protein extract. The reaction of dismutation was initiated by the addition of H_2O_2 (10 mM) as described by Aebi [83].

Transcriptome profiling

Gene expression data were extracted from the transcriptomic profiling experiment registered as E-MEXP-411 in

ArrayExpress [18,84]. Genes with a Bonferroni *P*-value higher than 5% were considered as being not differentially expressed as described by Lurin et al. [85]. Differentially expressed genes are those genes showing at least one *P*-value ≤ 0.05 after Bonferroni correction, in one of the MA/M, SA/M or S/M comparisons [18]. This *P*-value corresponds to genes whose Log_2(ratio) was greater than 0.73 or lower than -0.73 (corresponding to 1.6586-fold changes). This transcriptomic experiment compared the RNA profiles of three-week-old MS-grown plantlets transferred for 24 hours to the different control and treatment media described above (M, S, MA and SA).

Statistical analysis

Statistical analysis was carried out with the Minitab® 15.1.1.0 software (Minitab SARL, Paris, France). The non-parametrical Mann-Whitney test was used for the different comparisons of means. Means that were not significantly different ($P > 0.05$) show the same letter in graph representations.

Abbreviations

APX: ascorbate peroxidase; CAT: catalase; DAB: diaminobenzidine; DHAR: dehydroascorbate reductase; GR: glutathione reductase; H_2O_2: hydrogen peroxide; $HO^{\cdot}$: hydroxyl radical; MDAR: monodehydroascorbate reductase; MS: Murashige and Skoog; NBT: nitroblue tetrazolium; O_2: molecular oxygen; $^1O_2$: singlet oxygen; $O_2^{\cdot-}$: superoxide radical; PSII: photosystem II; ROS: reactive oxygen species; SOD: superoxide dismutase; SOSG: Singlet Oxygen Sensor Green®; DW: dry weight.

Authors' contributions

FR, CS, MB, IC and GG conceived the study and designed experiments. FR, MB and GG performed the experiments. FR, CS, MB, IC and GG carried out analysis and interpretation of experimental data including statistical analyses. FR, CS, IC and GG wrote the manuscript. All authors read and approved the final manuscript.

Additional material

Additional file 1

Patterns of accumulation of singlet oxygen.

Singlet oxygen detections using the SOSG probe have been done on 3-week-old MS-grown* Arabidopsis thaliana *plantlets subjected to subsequent treatment (12, 24, 48 or 72 hours) with 80 mM mannitol (M), 80 mM sucrose (S), 80 mM mannitol plus 10 μM atrazine (MA) or 80 mM sucrose plus 10 μM atrazine (SA). Image analysis and quantification of fluorescence was performed using ImageJ software. *Changes in average intensities are shown as percentage of mean fluorescence intensity of MS-grown plantlets as control.*

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Additional file 2

Patterns of accumulation of superoxide radical.

Detections and quantification have been done on 3-week-old MS-grown* Arabidopsis thaliana *plantlets subjected to subsequent treatment (12, 24, 48 or 72 hours) with 80 mM mannitol (M), 80 mM sucrose (S), 80 mM mannitol plus 10 μM atrazine (MA) or 80 mM sucrose plus 10 μM atrazine (SA). Superoxide radical content was expressed as nmoles of reduced NBT per g DW.

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Additional file 3

Patterns of accumulation of hydrogen peroxide.

Detections and quantification have been done on 3-week-old MS-grown* Arabidopsis thaliana *plantlets subjected to subsequent treatment (12, 24, 48 or 72 hours) with 80 mM mannitol (M), 80 mM sucrose (S), 80 mM mannitol plus 10 μM atrazine (MA) or 80 mM sucrose plus 10 μM atrazine (SA). Hydrogen peroxide content was expressed as μ moles of H_2O_2 per g DW.

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